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☐ *Additional inventors are being named on the ___ separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

Methods for Amplification of Nucleic Acid Sequences

Direct all correspondence to: **CORRESPONDENCE ADDRESS**

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Respectfully submitted,

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Methods for Amplification of Nucleic Acid Sequences

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Background of the Invention

Methods for the amplification of nucleic acid sequences are currently important tools in molecular biology. Frequently, a nucleic acid is present in extremely minute quantities in a given sample, making it advantageous to provide techniques for amplification of the nucleic acid to sufficient amounts to facilitate its further analysis.

Several such amplification techniques are currently employed in the art. In one well known method, for example, disclosed by Eberwine and his colleagues, a compound oligonucleotide is utilized for the amplification, wherein the compound oligonucleotide is provided with both a T7 promoter and a primer. (See e.g. U.S. Patent Nos. 5,716,785; 5,891,636; 5,958,688; and 6,291,170 B1; all of which are fully incorporated herein by reference).

Briefly summarized, in the Eberwine method, a cDNA copy is created of an initial mRNA, with subsequent second strand synthesis to create a cDNA which is double stranded. Amplification is conducted via the promoter from the compound oligonucleotide, with transcription proceeding off of the cDNA's second strand. Since the second strand is used for transcription, the Eberwine method produces amplified RNA which is anti-sense to the initial mRNA sequence. As a result, it produces materials which are the wrong sense for use on oligonucleotide arrays, when using conventional labeling

methods (i.e. reverse transcription and dye incorporation) and not incorporating the label into the aRNA.

Furthermore, since transcription occurs in Eberwine in the 3' to 5' direction with respect to the initial mRNA sequence, the method is, by its nature, biased toward that 3' end of the initial mRNA sequence, the end where priming occurs and from where transcription begins. In the Eberwine method, if the second strand template is not completely intact, a bias can be introduced into the results, favoring messages at the 3' end of the initial mRNA sequence, with loss of messages at the 5' end. For example, if second strand synthesis does not proceed to completion, messages corresponding to the 5' end of the initial mRNA will not be transcribed, resulting in an incomplete copy of the initial message. Thus, in general, the method may result in amplification wherein not every message is duplicated during the amplification procedure.

Accordingly, it would be advantageous to provide an improved method in the art for amplification of nucleic acid sequences. It would be particularly advantageous to provide a method which produces amplified nucleic acid of the same sense as the initial nucleic acid sample. It would further be advantageous to provide a method of amplification in which the copying of the amplified sequences results in a messages having complete fidelity to both the 5' and 3' ends of the initial nucleic acid sequence.

Summary of the Invention

It is an object of the present invention to provide an improved method for amplification of nucleic acid sequences.

It is a further object of the present invention to provide a method for amplification of DNA and RNA.

It is a further object of the present invention to provide a method for producing amplified sense nucleic acid from an initial nucleic acid sample.

It is a further object of the present invention to provide a method for amplification of nucleic acids which provides high fidelity to both the 5' and 3' end of the initial nucleic acid sequence.

It is a further object of the present invention to provide a method for amplification of nucleic acid, wherein the nucleic acid copying in the amplification step proceeds in a 5' to 3' direction relative to the initial nucleic acid sample.

Further objects and advantages of the invention will become apparent in conjunction with the detailed disclosure provided herein.

In accordance with the present invention, methods are provided for the amplification of nucleic acid sequences. The methods are particularly useful for the amplification of minute quantities of RNA (e.g. messenger RNA, ribosomal RNA, amplified RNA, transfer RNA) and/or DNA. Further to the invention, amplified copies are produced of the initial nucleic acid, wherein the amplified copies are all sense copies of the initial nucleic acid from the sample. As a result, in the case of mRNA for example, a method is provided allowing synthesis of large quantities of amplified RNA each including a sequence identical to that of the mRNA initially present in the sample in trace amounts.

Further in accordance with the invention, methods are provided for amplification of nucleic acid wherein priming occurs from one end of the nucleic acid strand and transcription occurs from the other end of the strand, to ensure the highest fidelity possible between the initial nucleic acid strand and the amplified copy. In the preferred embodiments, priming is conducted from the 3' end of the initial nucleic acid with a promoter subsequently added to a sequence corresponding to the 5' end of the initial nucleic acid, such that priming and nucleic acid synthesis initially occurs in the 3' to 5' direction, subsequently followed by transcription in the 5' to 3' direction relative to the initial nucleic acid sequence of interest.

In accordance with one embodiment of the preferred method, an existing mRNA sequence is used to create a single stranded cDNA. For example, in one preferred embodiment, a dT primer can be hybridized to the mRNA's 3' poly A tail and extended to form a double stranded mRNA/cDNA molecule, followed by degradation of the mRNA strand. Alternatively, any other suitable method for creation of a single stranded cDNA from the mRNA can be used. For example a random primer or a gene specific primer can be used to form the double stranded mRNA/cDNA molecule, followed by degradation of the mRNA.

Upon creation of the single stranded cDNA molecule (ss cDNA), a dA tail is attached to the molecule using any suitable protocol. In the preferred embodiment, a dA tail is attached to the 3' end using the enzyme Terminal deoxynucleotidyl Transferase (TdT).

Alternatively, a tail of another specifically desired sequence can be added to the 3' end. In the event that a tail other than a dA tail is added, the subsequent steps using a dT sequence complementary to the dA tail (for hybridization to that tail), are modified to include the appropriate sequence instead of dT, i.e. to use the sequence complementary to the tail that has been attached. Or, in the event that the sequence at the 5' end of the initial nucleic acid is known, addition of the tail to the 3' end can be skipped, if desired. When the sequence at the 5' end is known, the complement to that sequence can be treated as equivalent to the tail for further steps; the complement being present at the 3' end of the cDNA in the case of RNA as the initial nucleic acid, for example. However, addition of a tail (whether dA or otherwise) is preferred and will generally be necessary due to the fact that, in most applications, the sequence of the 5' end of the initial nucleic acid is unknown.

As an alternative to the use of mRNA, a strand of DNA can be used as the initial nucleic acid from the sample. In this embodiment, a random primer is used to create a complementary strand to the initial strand of DNA (the complementary strand being analogous to the cDNA described above), with a tail then being added to the complementary strand (e.g. through attachment to the 3' end by TdT) for use in the same manner as the dA tailed cDNA described herein (or with use of an existing sequence as the tail, as previously discussed).

Once the tailed nucleic acid has been produced (or a molecule is used with an existing sequence used as the tail), a promoter is placed on the 3' end by any of various suitable methods. The promoter is used to initiate transcription off of the 3' end of the tailed molecule. In other words, transcription proceeds off of the dA sequence, in the example when a dA tail is used. This transcription, proceeding

in the 3' to 5' direction with respect to the molecule with the promoter, corresponds to copying in the 5' to 3' direction with respect to the initial nucleic acid of interest. By the use of multiple rounds of transcription from the promoter, large numbers of copies are created of the initial nucleic acid, the copies being referred to as amplified mRNA or "Amp-mRNA" herein (when the initial nucleic acid is RNA), or as amplified DNA (when the initial nucleic acid is DNA).

Each of the amplified copies is identical in sense to the initial nucleic acid message. For example, in the case of an initial mRNA, since the multiple transcripts/amplified copies are all complementary to the cDNA, which is itself complementary to the initial mRNA, the amplified mRNA copies all contain the identical message to the initial mRNA sequence; i.e. amplified sense copies of the initial mRNA are produced, and not anti-sense copies.

These sense copies of amplified nucleic acid can then be used in any manner in which nucleic acids are used in the art, including any current, past, or future protocols.

In the case of amplified mRNA, in preferred embodiments, the amplified copies are converted to amplified cDNA copies, which can then be used in desired assays. In further preferred embodiments, the amplified nucleic acids are used in assays using microarrays. In alternative or additional embodiments of the invention, the amplified nucleic acids are used in labeled with dendritic reagents, particularly dendrimers such as 3DNA™ reagents available from Genisphere, Inc. of Montvale, New Jersey. Dendritic reagents are further described in Nilsen et al., Dendritic Nucleic Acid Structures, J. Theor. Biol., 187, 273-284 (1997); in Stears et al., A Novel, Sensitive Detection System for High-

Density Microarrays Using Dendrimer Technology, Physiol. Genomics, 3: 93-99 (2000); and in various U.S. patents, such as U.S. Patent Nos. 5,175,270; 5,484,904; 5,487,973; 6,072,043; 6,110,687; and 6,117,631, these references all being fully incorporated herein by reference.

Brief Description of the Figures

Figure 1 is a schematic illustration of a preferred method for amplification of nucleic acids in accordance with present invention, using staggered ligation to a tailed cDNA followed by nucleic acid amplification.

Figure 2 is a schematic illustration of an additional preferred method for amplification of nucleic acids in accordance with the present invention, using blunt ligation to a tailed cDNA, followed by nucleic acid amplification.

Figure 3 is a schematic illustration of a further additional preferred method for amplification of nucleic acids using hybridization of a complex primer to a tailed cDNA followed by nucleic acid amplification.

Detailed Description of the Invention and the Preferred Embodiments

In accordance with the present invention, methods are provided for the amplification of nucleic acid sequences. In the preferred embodiments, methods are provided for the amplification of DNA or RNA to produce amplified sense copies of the initial sequence. Further in the preferred embodiments, methods are provided wherein the amplification step (i.e. the step in which the amplified copies are

generated) utilizes nucleic acid copying commencing from the end corresponding to the 5' end of the initial sequence, via transcription in the 5' to 3' direction with respect to the initial nucleic acid strand.

In the preferred embodiments, a dA tailed cDNA copy is produced from the initial RNA strand. (Alternatively, a strand of DNA can serve as the initial nucleic acid, with a complement being created that is tailed and then used in the same manner as the dA tailed cDNA described herein). A promoter is then placed on the 3' dA tail, with the promoter being used to initiate multiple rounds of transcription to produce amplified sense copies of the initial nucleic acid, with transcription proceeding off of the dA tailed strand. Thus, as opposed to the Eberwine method, whether or not the second strand is intact will not impact the fidelity of the amplified transcript, so that loss of message is minimized. In fact, second strand synthesis is optional in the procedures of the present invention, as shown for example, in Figure 1 attached hereto, so that the present invention is effective even if the second strand is not synthesized. In addition, the methods of the present invention require significantly less time than the Eberwine method. Whereas, the Eberwine protocol normally takes several days, the present process can be done in one day if desired, or two if it is desired to conduct it overnight.

The amplified DNA or RNA can then be used in any desired context or protocol. Where the amplified nucleic acid is amplified mRNA, direct use of the amplified mRNA in further assays can be conducted for example, following such methods as disclosed in PCT Application Serial No. PCT/US01/22818 filed July 19, 2001 (Int'l Publication No. WO 02/06511), which is fully incorporated herein by reference.

Alternatively, the amplified mRNA can be used to create amplified cDNA, which is used for further analysis (with cDNA often being preferred in the art, due to its greater stability). It is further preferred that the cDNA be utilized in assays using oligonucleotide microarrays due to their high efficiency, providing the ability to conducts thousands of test-tube like experiments virtually simultaneously. The cDNA can be directly labeled if desired, or can be indirectly labeled for its further analysis. One preferred labeling method utilizes capture sequences for linkage of the cDNA to a labeled dendrimer, as disclosed for example in PCT Application Serial Number PCT/US01/07477 filed March 8, 2001 (Int'l Publication No. WO 01/066555), PCT Application Serial No. PCT/US01/22818 filed July 19, 2001 (Int'l Publication No. WO 02/06511), and PCT Application Serial No. PCT/US01/29589 filed September 20, 2001 (Int'l Publication No. WO 02/033125), all of which are fully incorporated herein by reference. It is further preferred that a blocker be used consisting of Locked Nucleic Acid ("LNA"), to minimize nonspecific hybridizations, as disclosed for example in PCT/US02/27799 filed 3 September 2002 (Int'l Publication No. WO 03/020902), which is fully incorporated herein by reference.

The foregoing discussion discloses and describes merely exemplary embodiments of the present invention. One skilled in the art will readily recognize from such discussion, and from the accompanying drawings and examples, that various modifications and variations can be made therein without departing from the spirit and scope of the invention. The present application is intended to cover all such modifications and variations.

In accordance with the present invention, several methods illustrating preferred embodiments of the invention are provided as discussed below.

Method 1: Staggered ligation to tailed cDNA, followed by T7 Amplification

In accordance with a one preferred embodiment of the present invention, the method for amplification of nucleic acid sequences can be conducted as shown, for example, in Figure 1. In accordance with the method, staggered ligation of a promoter to a tailed cDNA (created from an initial mRNA sequence) is followed by amplification from the promoter to produce amplified copies of the initial mRNA sequence.

As shown in Figure 1, a sample is provided having mRNA therein (the "initial mRNA" of Figure 1), the mRNA having a poly A tail at its 3' end. Alternatively, an mRNA can be utilized lacking such a tail, with the tail being added on prior to commencement of the present method. Or, a random primer or a gene specific primer can be used to reverse transcribe the nucleic acid (whether the nucleic acid has a a poly A tail or not), in place of hybridization of a dT primer to a poly A tail. The initial molecule with is also referred to herein as "nucleic acid a", as shown in Figure 1, the references nucleic acid a, b, c etc. (or molecules a, b, c, etc.) being provided as alternative terms for convenience of reference.

In a first step of the method shown in Figure 1, a dT primer is added to the sample for hybridization to the initial mRNA 3' poly A tail, and synthesis is conducted using DNA polymerase to produce a double stranded mRNA/cDNA molecule ("nucleic acid b"), i.e. a double stranded molecule

having the initial mRNA hybridized to a complementary cDNA. (Or, as discussed above, a random primer or gene specific primer can be used in place of the dT primer, to create the molecule of mRNA/cDNA). The mRNA strand of this duplex is then degraded, leaving a single stranded cDNA molecule ("nucleic acid c"). In the case of use of a dT primer, the single stranded cDNA ("ss cDNA") has a 5' end having a poly dT or dU tail, the 5' dT (or dU) tail having originated from the primer. Likewise, in the case of the use of another primer, that primer will remain at the 5' end of the single stranded cDNA.

In step 3, this single stranded cDNA is modified to add a dA tail thereto (this step also being referred to herein as "tailing"). In the preferred embodiment, the enzyme Terminal deoxynucleotidyl transferase (TdT) is used to add a dA tail to the 3' terminus of the cDNA ("TdT/dA tailing"), TdT being a known DNA polymerase catalyzing the terminal addition of deoxyribonucleotides to the 3'-OH termini of DNA. At the end of this step, a cDNA molecule has been produced ("nucleic acid d") which is complementary to the initial mRNA sequence, and which has a 3' dA tail attached thereto.

In step 4 of the method shown in Figure 1, a promoter is attached to the dA tailed cDNA's 3' end. In a preferred embodiment, the T7 promoter is utilized, although other suitable promoters can alternatively be used consistent with the present invention. In the method of Figure 1, staggered ligation is conducted of a double stranded T7 promoter to the 3' end of the tailed cDNA. Alternatively, other methods may be used for attachment of the promoter to the tailed cDNA, including, but not limited to those described below, and shown in Figures 2 and 3.

In the method of staggered ligation shown in Figure 1 (step 4), a double stranded ("ds") promoter is provided having a dT overhang, with the dT overhang hybridizing the double stranded T7 promoter to the cDNA's 3' dA tail. The 3' dA tail of the dA tailed cDNA molecule, therefore, provides a partial overlapping complementary sequence to the ds T7 promoter, thereby aligning the T7 promoter for ligation. Using a ligase, the double stranded T7 promoter is ligated to the dA tailed cDNA molecule, so that the dA tailed cDNA now carries a promoter on its 3' end ("nucleic acid e").

Following ligation of the promoter onto the dA tailed cDNA, a large number of mRNA messages can be transcribed off of the cDNA, as shown in Figure 1, step 5. For example, when the T7 promoter is used, T7 polymerase and NTPs can be added to transcribe messages off of the cDNA template, as shown in step 5 of Figure 1. In the method of Figure 1, transcription can be conducted off of a single stranded cDNA provided that the double stranded promoter is affixed thereto. Alternately, in alternative embodiments, transcription can be conducted from a double stranded molecule, as shown, for example, in the method of Figure 2.

Thus, in step 5 (the amplification step), repeated cycles of transcription are conducted to create large numbers of copies of the initial mRNA in the sample, the copies being referred to herein as amplified mRNA ("Amp-mRNA"; "nucleic acid f" in Figure 1). The amplified mRNA copies include a sequence identical to the mRNA initially present in the sample and, furthermore, are sense copies of the initial mRNA, not antisense. Furthermore, transcription is conducted in the 3' to 5' direction with respect to the dA tailed cDNA, which corresponds to copying in the 5' to 3' direction with respect to the initial mRNA sequence.

As a result of this procedure, amplified mRNA is generated, providing a large number of copies of the mRNA that was initially present in trace amounts. As shown in the figure, the amplified mRNA copies each include a sense copy of the initial mRNA sequence with a dA tail, and include a minor additional sequence added at the 5' end. More generally, the amplified RNA (nucleic acid f) is a copy identical to the initial RNA "nucleic acid a" (whether that initial RNA had a poly A tail or not), with the copy having a small addition in the form of a short sequence at the 5' end. In the case shown in the figure, for example, the 5' end addition includes a short sequence transcribed from the portion of the promoter ligated to the dA tailed cDNA strand, followed by a dT sequence transcribed from the 3' dA tail of the cDNA.

Following the steps provided above, the amplified mRNAs so produced can be used in any manner and for any purpose that the initial mRNA could have been used, albeit now advantageously present in greater quantities. For example, the amplified mRNA can be in accordance with any of the remaining steps of method 1 of the invention, or in any other desired method or for any other desired purpose, whether any existing method, or any method to be developed in the future.

In further steps of the invention, preferred methods are disclosed for use of the amplified mRNA in various experimental procedures. However, as discussed above, the further assay procedures shown and described in Figure 1 are merely exemplary of one preferred embodiment, as it will be understood that any desired protocols can be provided for the use of the amplified mRNA.

In the preferred embodiment of steps six onward of Figure 1, the amplified copies of mRNA

are used to created cDNA (amplified cDNA). This amplified cDNA is used for subsequent assay (preferably in array experiments).

Alternatively, instead of creating cDNA copies, as shown in Figure 1, the amplified mRNA could itself be assayed directly if desired. While the mRNA copies are usually of insufficient stability for use in the further assay procedure, direct use of the mRNA is, nonetheless, possible, as described for example in PCT Application Serial No. PCT/US01/22818 filed July 19, 2001 (Int'l Publication No. WO 02/06511), which is fully incorporated herein by reference.

Steps six and seven of Figure 1, illustrate one example of the use of the amplified mRNA to create cDNA copies. As shown therein, using reverse transcriptase, cDNAs are transcribed off of the amplified mRNAs to generate a double stranded cDNA/mRNA molecule ("nucleic acid g"), with subsequent degradation of the mRNA strands to yield single stranded amplified cDNAs ("Amp-cDNA", nucleic acid h). In one preferred embodiment, as shown in step six of Figure 1, a random primer is used (e.g. Genisphere Array 350 Random Primer, available from Genisphere Inc. of Montvale, New Jersey), so that variable length amplified cDNAs are produced, these variable length cDNA ranging from small fragments of the amp-mRNA to the full size of the mRNA message.

Following creation of the double stranded molecule, the strand containing the amplified mRNA is degraded, leaving single stranded copies of amplified cDNA (nucleic acid h), the cDNA copies being of varying lengths when a random primer is utilized.

These amplified cDNAs can then be used in a desired experiment or assay. Any desired assay means can be used. In the preferred embodiment, the assay uses a microarray for simultaneously conducting a large number of large scale "test-tube" like experiments.

Likewise, any desired labeling means can be used for detection of the cDNAs in the assay. For example, the cDNAs can be produced using radioactive nucleotides, for labeling and use of the cDNAs in subsequent experiments.

In a preferred embodiment, as shown in step 8 of Figure 1, a capture sequence is ligated onto the end of each amplified cDNA, the capture sequence being complementary to a label molecule. Examples of such labeling methods using capture sequences are disclosed in the PCT Applications previously cited herein and cited below, all of which are fully incorporated herein by reference.

When the cDNA is applied to the assay medium (e.g. to a microarray surface), the label molecule having the complement to the capture sequence is used to detect the hybridization of the cDNA to the array. Any desired molecule can be used for the label. In preferred embodiments, the label molecule is a labeled dendrimer to provide increased labeling efficiency. Dendrimers and the use of dendrimers is described in Nilsen et al., Dendritic Nucleic Acid Structures, J. Theor. Biol., 187, 273-284 (1997); in Stears et al., A Novel, Sensitive Detection System for High-Density Microarrays Using Dendrimer Technology, Physiol. Genomics, 3: 93-99 (2000); and in various patents and patent applications, such as U.S. Patent Nos. 5,175,270; 5,484,904; 5,487,973; 6,072,043; 6,110,687; 6,117,631, PCT Application Serial Number PCT/US01/07477 filed March 8, 2001 (Int'l Publication

No. WO 01/066555), PCT Application Serial No. PCT/US01/22818 filed July 19, 2001 (Int'l Publication No. WO 02/06511), and PCT Application Serial No. PCT/US01/29589 filed September 20, 2001 (Int'l Publication No. WO 02/033125), all of which are fully incorporated herein by reference.

In a further preferred embodiment of the invention, Locked Nucleic Acid ("LNA") is used as a blocker to prevent non-specific hybridizations, as further discussed in PCT Application Serial No. PCT/US02/27799 filed 3 September 2002 (Int'l Publication No. WO 03/020902), which is fully incorporated herein by reference. For example, as shown in step 9 of Figure 1, the LNA blocker can be used to hybridize to the addition located at the 3' end of the amplified cDNA to minimize any nonspecific binding effects when the amplified cDNA is applied to the assay medium.

Any desired method can then be used to conduct the desired assay. For example, in one preferred embodiment, a two step hybridization can be conducted as shown in step 10 of Figure 1. In a two step hybridization, hybridization of the cDNA to the array and hybridization of the label molecule to the cDNA (via the capture sequence) are conducted as two separate steps. Alternatively, a one step hybridization can be conducted as discussed in PCT Application Serial Number PCT/US01/07477 filed 8 March 2001 (Int'l Publication No. WO 01/066555), which is fully incorporated herein by reference.

A hybridization pattern is then generated, and signal detection is conducted to view and then analyze the assay results.

Method 2 - Blunt Ligation of T7 Promoter

In accordance with an alternative method for amplification of nucleic acid sequences, blunt ligation of a T7 promoter to a dA tailed cDNA is conducted, as shown for example, in Figure 2.

In this method, a user begins with a nucleic acid sequence (preferably mRNA), and using that sequence generates a 3' dA tailed cDNA, in the same manner as previously described above with respect to method 1.

Following creation of the dA tailed cDNA, a dT primer is hybridized onto the 3' dA tail, as shown in step 4 of Figure 2. Using DNA polymerase and dNTPs, a double stranded cDNA is then generated("nucleic acid f" of Figure 2), the double stranded molecule being provided with a blunt end at the end corresponding to the 3' dA tailed cDNA, as a result of the exonuclease activity of the DNA polymerase.

Using DNA ligase, a double stranded T7 promoter is then ligated onto the double stranded cDNA (producing "nucleic acid g"). The promoter is ligated onto the end of the ds cDNA to which the 3' dA tail had been added, as shown in Step 5 of Figure 2.

T7 polymerase and NTPs are then used to transcribe mRNA messages from the double stranded cDNA, producing amplified mRNA ("nucleic acid h" of Figure 2). In other words, amplified sense copies of the original mRNA are produced, with transcription having been conducted in a direction corresponding to the 5' to 3' direction of the initial mRNA.

The amplified mRNAs, can now be used in any desired method and for any desired purpose, as previously discussed. In preferred embodiments, amplified cDNAs can be created, e.g. for subsequent microarray assays as discussed with respect to Figure 1; or, alternatively, any other desired protocol can be conducted.

Method 3 - Complex Primer - Alternative T7 Amplification

In accordance with a further alternative method for amplification of nucleic acid sequences, a method for amplification using a complex primer is conducted, as shown for example, in Figure 3.

In this method, a user begins with a nucleic acid sequence (preferably mRNA), and using that sequence generates a 3' dA tailed cDNA, in generally the same manner as previously described above. As shown in Figure 3, a dT primer can be used as previously discussed. Alternatively, in this step a random primer or gene specific primer can be used, as discussed in conjunction with Method 1. Likewise, a random primer or gene specific primer can be alternately utilized in method 2 described above, as well.

Once the dA tailed cDNA has been produced, a single strand of a T7 promoter having a dT tail is hybridized to the 3' dA end of the cDNA, as shown in step 4 of Figure 3. Using DNA polymerase and dNTPs, the single stranded cDNA with attached single stranded promoter is converted to a double stranded molecule, including conversion of the single stranded T7 promoter to a double stranded T7 promoter.

Subsequently, in step 5, using T7 polymerase and NTPs, amplified mRNA is produced from the double stranded cDNA molecule. The amplified mRNA is of the same sense as the initial mRNA, and is produced using transcription in a direction corresponding to the 5' to 3' direction of the initial mRNA strand. As discussed above, this amplified mRNA can be used in any method or context in which mRNA is utilized in molecular biological protocols. For example, the amplified mRNA can be used with the methods shown in steps 6 onward of Figure 3, and/or in any of the methods described or illustrated with respect to Methods 1 and 2 (and corresponding Figures 1 and 2).

Having described the invention with respect to several preferred embodiments, several illustrative examples of protocols using the present inventions are provided below.

Example 1:

Opposite Strand T7-Amplification (Oligo Array Compatible) using Staggered Ligation

First Strand Synthesis:

a. Reverse transcribe up to 10ug of total RNA using cap03 Genisphere RT primer in a total volume of 20ul. (Superscript II or Genisphere RT Reagents may be used). Note: For RNA amounts greater than 5ug, use 5pmol/ul RT primer. Standard dT and/or RP primers and/or standard RT primers may be used instead of Genisphere primers.

1-10ul total RNA
1ul 1pmol/ul or 5pmole/ul Genisphere RT primer

11ul

Heat to 80°C for 10 minutes. Ice immediately for 1-2minutes.

Add RT components using one of the following options:

4ul SSII 5X 1st Strand Buffer	4ul 5X Rxn Buffer (Promega)
2ul DTT	2ul nuclease free water (Vial 10)
1ul dNTP (Genisphere 10mM ea)	1ul dNTP mix (Vial 3)
1ul Superasin	1ul RNase Inhibitor (Vial 4)
1ul Superscript II	1ul MMLV RT (Promega)
20ul final volume	

Incubate at 42°C for 1.5-2 hours.

b. Stop reaction with 3.5ul of 0.5M NaOH/50mM EDTA and heat to 65°C for 10 minutes.

c. Neutralize the reaction with 5ul of 1M Tris-HCl, pH 7.5.

d. Adjust volume to 50ul with 1X TE pH 8.0.

e. Purify the reaction using Qiagen QIAquick PCR purification kit as directed by the manufacturers protocol. Elution will be in 50ul.

TdT Tailing of 1st Strand cDNA:

Protocol Including Qiagen Purification of TdT Reaction:

a. Heat cDNA to 80°C for 5-10 minutes to denature. Cool on ice immediately for 1-2 minutes.

[redacted]

b. Tail cDNA with dATP mix to about 20-50bp length as described below, and incubate the reaction at 37°C for 10-60 minutes (TBD).

50ul cDNA
14ul 5X TdT buffer
2ul dATP mix, no ddATP (~10mM dATP stock)
2.5ul water
1.5ul TdT

70ul

Alternatively: Tris based buffer may be used in place of standard TdT buffer avoid purification prior to ligation (see Protocol Excluding Purification, on following page).

c. Stop the reaction with 8ul 0.5M EDTA. Add 22ul 1X TE pH 8.0 to adjust the volume to 100ul.

d. Purify the reaction using Qiagen QIAquick PCR purification kit as directed by the manufacturers protocol. Elution will be in 50ul.

e. Heat to 95-100°C for 10-15 minutes to denature cDNA, ice immediately for 1-2 minutes.

f. Add T7 promoter ligation components as follows and incubate reaction at room temperature for 30-60 minutes.

50ul tailed cDNA
10ul 6X Ligation Mix (containing T7 promoter sequence)
2.5ul T4 DNA Ligase

62.5ul

[redacted]

g. Add 7ul 0.5M EDTA. Optional: Heat to 80°C for 10minutes. Add 30.5ul of 1X TE pH 8.0 to adjust final volume to 100ul.

h. Purify the reaction using Qiagen QIAquick PCR purification kit as directed by the manufacturers protocol. Elution will be in 50ul.

Protocol Excluding Qiagen Purification of TdT Reaction:

a. Heat cDNA to 80°C for 5-10 minutes to denature. Cool on ice immediately for 1-2 minutes.

b. Tail cDNA with dATP mix to about 20-50bp length as described below, and incubate the reaction at 37°C for 10-60 minutes (TBD).

50ul cDNA
6ul 10X Tris-based buffer
2ul dATP mix, no ddATP (~10mM dATP stock)
1ul water
1ul TdT

60ul

c. Incubate at 95-100°C for 10-15 to inactivate TdT enzyme and denature cDNA, ice immediately for 1-2 minutes. (DO NOT USE EDTA!)

d. Add ligation components as follows and incubate reaction at room temperature for 30-60 minutes.

60.0ul tailed cDNA
12.5ul 6X Ligation Mix
2.5ul T4 DNA Ligase

75.0ul

[redacted]

e. Add 8ul 0.5M EDTA. Optional: Heat to 80°C for 10minutes. Add 17ul of 1X TE pH 8.0 to adjust final volume to 100ul.

f. Purify the reaction using Qiagen QIAquick PCR purification kit as directed by the manufacturers protocol. Elution will be in 50ul.

Concentration of cDNA Containing T7 Promoter Sequences:

g. Ethanol precipitate cDNA to concentrate.

h. Resuspend pellet in nuclease free water. Heat to 80°C for 15-20 minutes, vortex.

i. Remove up to 2ug equivalent for IVT reaction. Note: When using Ambion messageAmp aRNA kit, aliquot must not exceed 8ul.

In Vitro Transcription:

a. Add in vitro transcription reagents and incubate reaction at 37°C for 6-14 hours (see below).

8ul purified cDNA (containing ligated T7 promoter)
2ul T7 ATP Soln (75mM)
2ul T7 CTP Soln (75mM)
2ul T7 GTP Soln (75mM)
2ul T7 UTP Soln (75mM)
2ul T7 10X Reaction Buffer
2ul T7 Enzyme Mix (including RNase inhibitor)

20ul (use Ambion reagents or equivalent)

b. Optional: Add 2ul DNase I to reaction and incubate for 30 minutes at 37°C.

c. Optional: Heat to 80°C for 10 minutes to inactivate DNase I. Note: DNase I will be removed during purification.

Purification of Amplified RNA Using Qiagen RNeasy Kit

a. Purify amplified RNA sample using Qiagen RNeasy kit (protocol for RNA cleanup) as directed by the manufacturer.

b. QC RNA sample by obtaining O.D. reading and visualizing sample on gel.

Example 2:
Opposite Strand T7-Amplification (Oligo Array Compatible) Using Blunt End Ligation

First Strand Synthesis:

a. Reverse transcribe up to 10ug of total RNA using cap03 Genisphere RT primer in a total volume of 20ul. (Superscript II or Ambion messageAMP aRNA Reagents may be used). Note: For RNA amounts greater than 5ug, use 5pmol/ul RT primer. Standard dT and/or RP primers and/or standard RT primers may be used instead of Genisphere primers.

1-10ul total RNA
1ul 1pmol/ul or 5pmole/ul Genisphere RT primer

11ul

Heat to 80°C for 10 minutes. Ice immediately for 1-2minutes.

Add RT components using one of the following options:

4ul SSII 5X 1st Strand Buffer	2ul 10X 1st Strand Buffer (Ambion)
2ul DTT	1ul nuclease free water (Ambion)
1ul dNTP (Genisphere 10mM ea)	1ul dNTP mix (Ambion)
1ul Superasin	1ul RNase Inhibitor (Ambion)
1ul Superscript II	1ul MMLV RT (Ambion)
20ul final volume	

Incubate at 42°C for 1.5-2 hours.

b. Stop reaction with 3.5ul of 0.5M NaOH/50mM EDTA and heat to 65°C for 10 minutes.

c. Neutralize the reaction with 5ul of 1M Tris-HCl, pH 7.5.

d. Adjust volume to 50ul with 1X TE pH 8.0.

e. Purify the reaction using Qiagen QIAquick PCR purification kit as directed by the manufacturers protocol. Elution will be in 50ul.

TdT Tailing of 1st Strand cDNA:

a. Heat cDNA to 80°C for 5-10 minutes to denature. Cool on ice immediately for 1-2 minutes.

b. Tail cDNA with dATP mix to about 50bp length as described below, and incubate the reaction at 37°C for 30-60 minutes.

50ul cDNA
14ul 5X TdT buffer
2ul dATP mix (50:1 ratio of 100mM dATP:ddATP)
2.5ul water
1.5ul TdT

70ul

c. Stop the reaction with 8ul 0.5M EDTA. Add 22ul 1X TE pH 8.0 to adjust the volume to 100ul.

d. Purify the reaction using Qiagen QIAquick PCR purification kit as directed by the manufacturers protocol. Elution will be in 50ul.

Second Strand Synthesis:

a. Add 2ul standard dT primer (about 100 pmol) to the purified first strand reaction. Heat the mixture to 95-100°C for 10 minutes to denature possible A/T circular hybrids. Ice immediately for 1-2 minutes.

b. Add remaining second strand reagents (see below) and incubate the reaction at 16°C for 2 hours.

50ul purified cDNA
22ul water
10ul 10X second strand buffer
2ul dT primer (added above)
4ul dNTP mix
2ul DNA polymerase I

100ul (use Ambion reagents or other equivalent)

[redacted]

c. Purify second strand reaction using Qiagen QIAquick PCR Purification kit as directed by the manufacturers protocol. Elution will be in 50ul.

Blunt End Ligation:

a. Add ligation components as follows and incubate reaction at room temperature for 60 minutes.

50.0ul 2nd strand cDNA
10.0 ul 6X Ligation Mix
2.5 ul T4 DNA Ligase

62.5 ul

b. Add 7ul 0.5M EDTA. Optional: Heat to 80°C for 10minutes. Add 30.5 ul of 1X TE pH 8.0 to adjust final volume to 100ul.

c. Purify the reaction using Qiagen QIAquick PCR purification kit as directed by the manufacturers protocol. Elution will be in 50ul.

d. Ethanol precipitate cDNA to concentrate.

e. Resuspend pellet in nuclease free water. Heat to 80°C for 15-20 minutes, vortex.

f. Remove up to 2ug equivalent for IVT reaction. Note: When using Ambion mcssageAmp aRNA kit, aliquot must not exceed 8ul.

In Vitro Transcription:

a. Add in vitro transcription reagents and incubate reaction at 37°C for 6-14 hours (see below).

8ul purified 2nd strand cDNA
2ul T7 ATP Soln (75mM)
2ul T7 CTP Soln (75mM)
2ul T7 GTP Soln (75mM)
2ul T7 UTP Soln (75mM)
2ul T7 10X Reaction Buffer
2ul T7 Enzyme Mix

20ul (use Ambion reagents or equivalent)

b. Optional: Add 2ul DNase I to reaction and incubate for 30 minutes at 37°C.

c. Optional: Heat to 80°C for 10 minutes to inactivate DNase I. Note: DNase I will be removed during purification.

Purification of Amplified RNA Using Qiagen RNeasy Kit

a. Purify amplified RNA sample using Qiagen RNeasy kit (protocol for RNA cleanup) as directed by the manufacturer.

b. QC RNA sample by obtaining O.D. reading and visualizing sample on gel.

Example 3:
Opposite Strand T7-Amplification (Oligo Array Compatible)
Using T7(dT) 24 Primer

First Strand Synthesis:

a. Reverse transcribe up to 10ug of total RNA using cap03 Genisphere RT primer in a total volume of 20ul. (Superscript II or Ambion messageAMP aRNA Reagents may be used). Note: For RNA amounts greater than 5ug, use 5pmol/ul RT primer. Standard dT and/or RP primers and/or standard RT primers may be used instead of Genisphere primers.

1-10ul total RNA
1ul 1pmol/ul or 5pmole/ul Genisphere RT primer

11ul

Heat to 80°C for 10 minutes. Ice immediately for 1-2minutes.

Add RT components using one of the following options:

4ul SSII 5X 1st Strand Buffer	2ul 10X 1st Strand Buffer (Ambion)
2ul DTT	1ul nuclease free water (Ambion)
1ul dNTP (Genisphere 10mM ea)	1ul dNTP mix (Ambion)
1ul Superasin	1ul RNase Inhibitor (Ambion)
1ul Superscript II	1ul MMLV RT (Ambion)

20ul final volume

Incubate at 42°C for 1.5-2 hours.

b. Stop reaction with 3.5ul of 0.5M NaOH/50mM EDTA and heat to 65°C for 10 minutes.

c. Neutralize the reaction with 5ul of 1M Tris-HCl, pH 7.5.

d. Adjust volume to 50ul with 1X TE pH 8.0.

e. Purify the reaction using Qiagen QIAquick PCR purification kit as directed by the manufacturers protocol. Elution will be in 50ul.

TdT Tailing of 1st Strand cDNA:

a. Heat cDNA to 80°C for 5-10 minutes to denature. Cool on ice immediately for 1-2 minutes.

b. Tail cDNA with dATP mix to about 50bp length as described below, and incubate the reaction at 37°C for 30-60 minutes.

50ul cDNA
14ul 5X TdT buffer
2ul dATP mix (50:1 ratio of 100mM dATP:ddATP)
2.5ul water
1.5ul TdT

70ul

c. Stop the reaction with 8ul 0.5M EDTA. Add 22ul 1X TE pH 8.0 to adjust the volume to 100ul.

d. Purify the reaction using Qiagen QIAquick PCR purification kit as directed by the manufacturers protocol. Elution will be in 50ul.

Second Strand Synthesis:

a. Add 2ul of T7-dT(24) primer (about 100pmol) to the purified first strand reaction. Heat the mixture to 95-100°C for 10 minutes to denature possible A/T circular hybrids. Ice immediately for 1-2 minutes.

b. Add remaining second strand reagents (see below) and incubate the reaction at 16°C for 2 hours.

50ul purified cDNA
22ul water
10ul 10X second strand buffer
2ul T7-dT(24) primer (added above)
4ul dNTP mix
2ul DNA polymerase I

100ul (use Ambion reagents or other equivalent)

c. Purify second strand reaction using Qiagen QIAquick PCR Purification kit as directed by the manufacturers protocol. Elution will be in 50ul.

d. Ethanol precipitate cDNA to concentrate.

e. Resuspend pellet in nuclease free water. Heat to 80°C for 15-20 minutes, vortex.

f. Remove up to 2ug equivalent for IVT reaction. Note: When using Ambion messageAmp aRNA kit, aliquot must not exceed 8ul.

In Vitro Transcription:

a. Add in vitro transcription reagents and incubate reaction at 37°C for 6-14 hours (see below).

8ul purified 2nd strand cDNA
2ul T7 ATP Soln (75mM)
2ul T7 CTP Soln (75mM)
2ul T7 GTP Soln (75mM)
2ul T7 UTP Soln (75mM)
2ul T7 10X Reaction Buffer
2ul T7 Enzyme Mix

20ul (use Ambion reagents or equivalent)

b. Optional: Add 2ul DNase I to reaction and incubate for 30 minutes at 37°C.

c. Optional: Heat to 80°C for 10 minutes to inactivate DNase I. Note: DNase I will be removed during purification.

Purification of Amplified RNA Using Qiagen RNeasy Kit

a. Purify amplified RNA sample using Qiagen RNeasy kit (protocol for RNA cleanup) as directed by the manufacturer.

b. QC RNA sample by obtaining O.D. reading and visualizing sample on gel.

The foregoing discussion, therefore, discloses and describes merely exemplary and preferred embodiments of the present invention. One skilled in the art will readily recognize from such discussion, and from the accompanying drawings and examples, that various changes, modifications and variations can be made therein without departing from the spirit and scope of the invention as

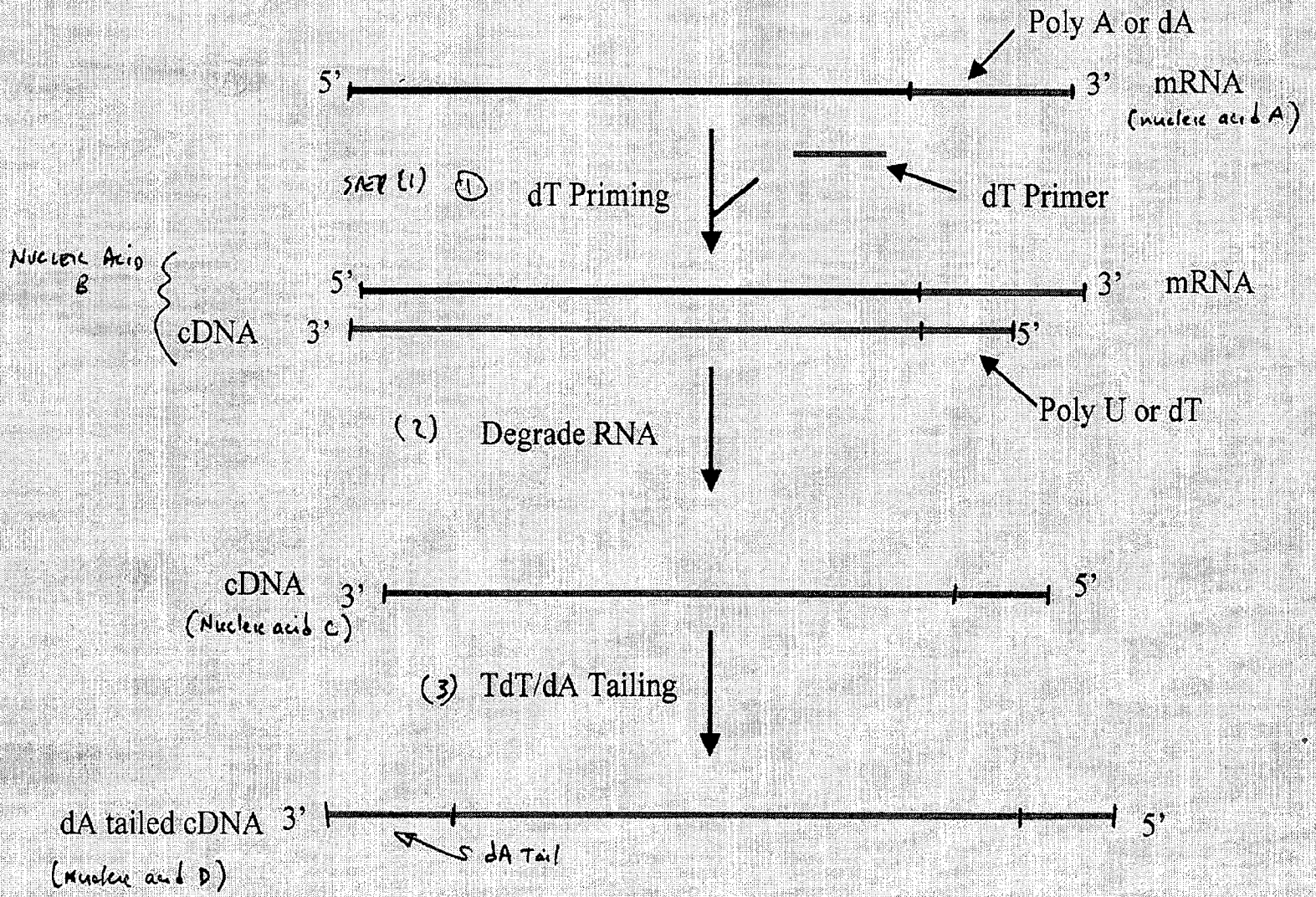
defined in the claims. One skilled in the art may likewise by applying current or future knowledge, adopt the same for use in accordance with the present invention. Yet, having described this invention with regard to specific embodiments, it is to be understood that the description is not meant as a limitation, and that the present application covers all such embodiments, modifications and variations of the presently disclosed inventions.

Claims

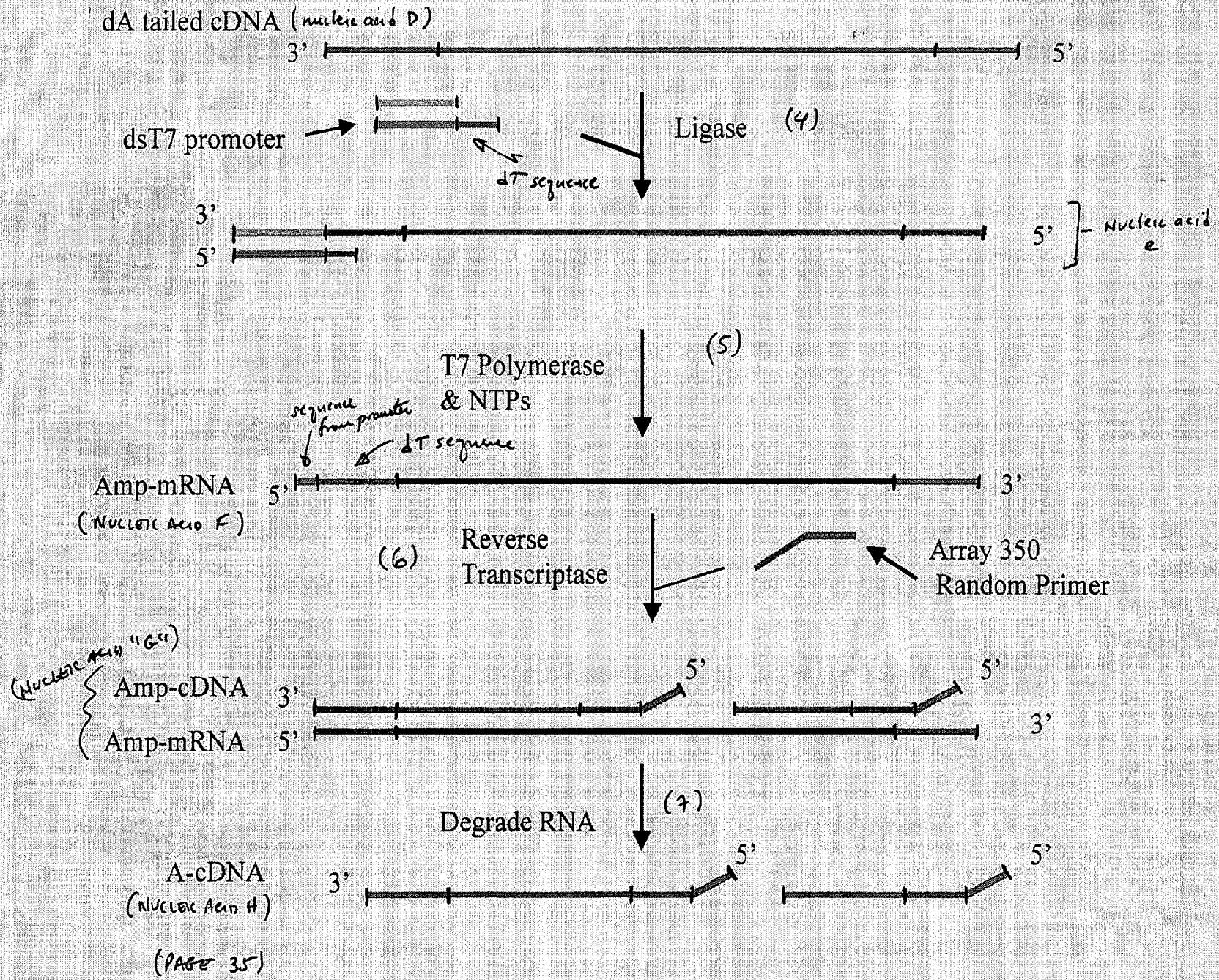
What is claimed is:

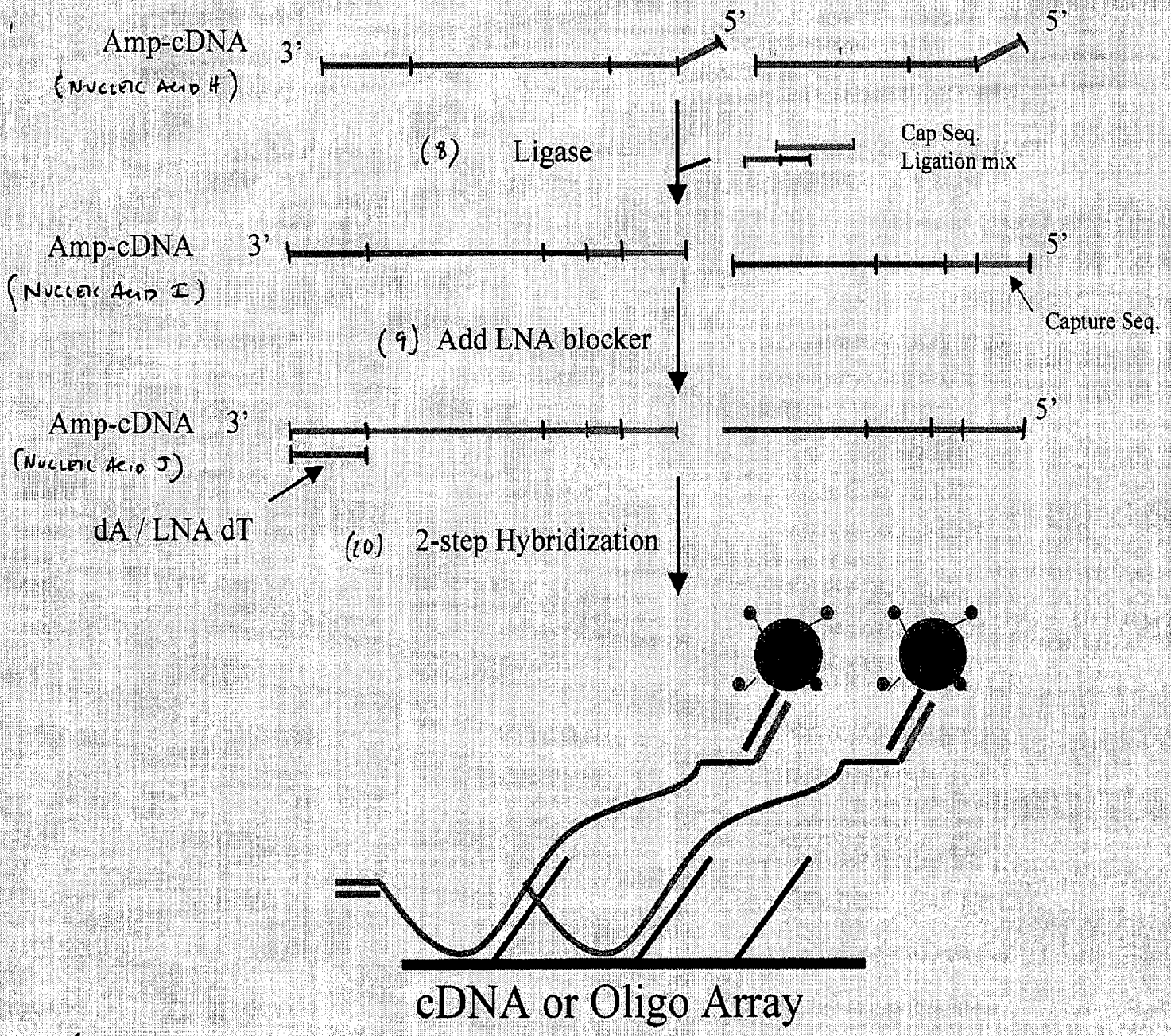
1. Methods for amplification of nucleic acid sequences having all of the features disclosed above.
2. Nucleic acid sequences produced by any of the methods for amplification of nucleic acid sequences disclosed above.

Figure 1: Staggered Ligation to tailed cDNA followed by T7 Amplification



(PAGE 34)


dA tailed cDNA (nucleic acid D)
3'
5'
dsT7 promoter
dT sequence
Ligase
(4)
3'
5'
5'
Nucleic acid E
(5)
T7 Polymerase
& NTPs
sequence from promoter
dT sequence
Amp-mRNA
(Nucleic Acid F)
5'
3'
(6)
Reverse
Transcriptase
Array 350
Random Primer
(Nucleic Acid "G")
Amp-cDNA
Amp-mRNA
3'
5'
5'
3'
5'
(7)
Degrade RNA
A-cDNA
(Nucleic Acid H)
3'
5'
5'
(PAGE 35)


Amp-cDNA
(Nucleic Acid H)
3'
5'
5'
(8) Ligase
Cap Seq.
Ligation mix
Amp-cDNA
(Nucleic Acid I)
3'
5'
Capture Seq.
(9) Add LNA blocker
Amp-cDNA
(Nucleic Acid J)
3'
5'
dA / LNA dT
(10) 2-step Hybridization
cDNA or Oligo Array

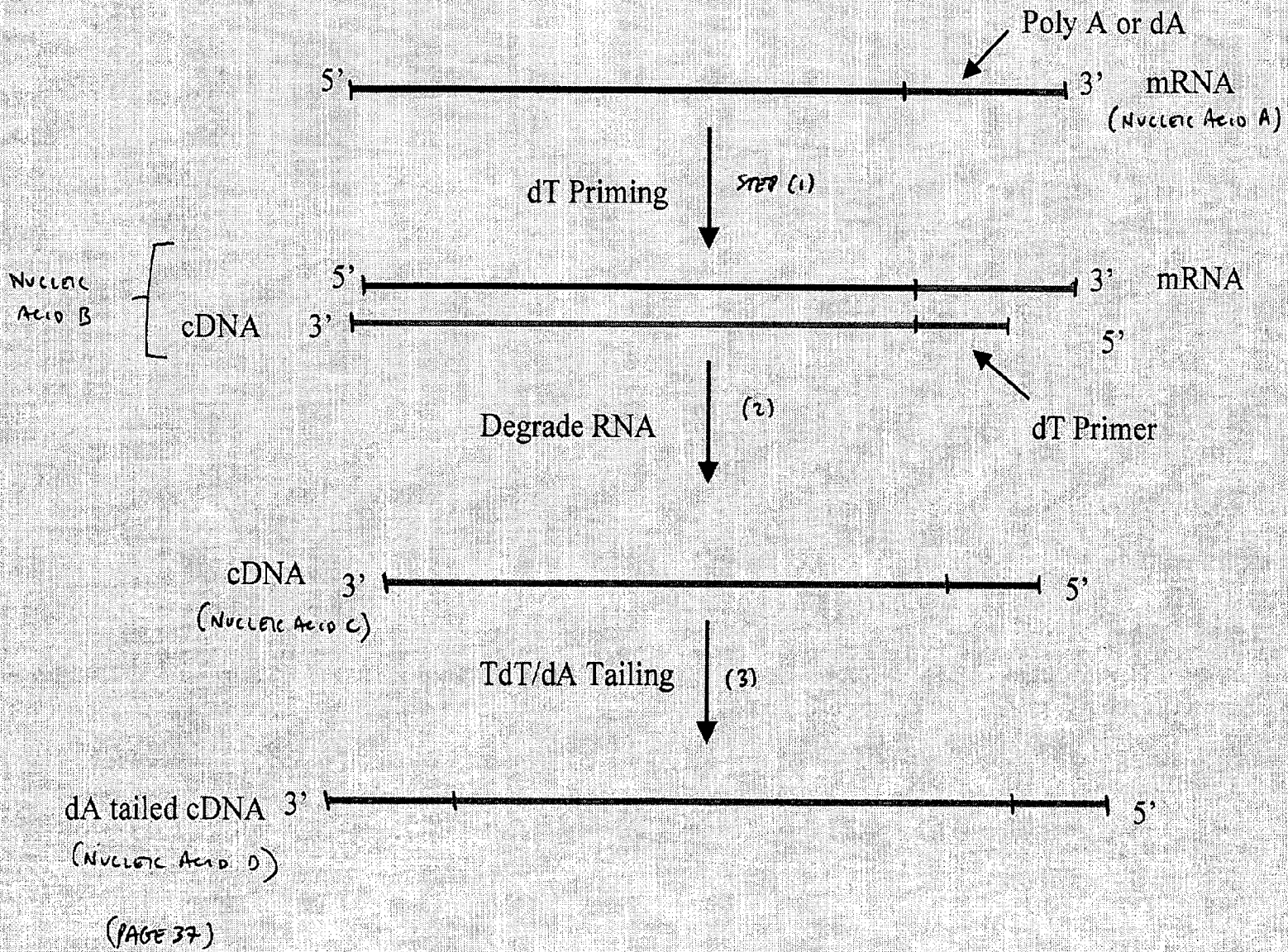
(PAGE 36)

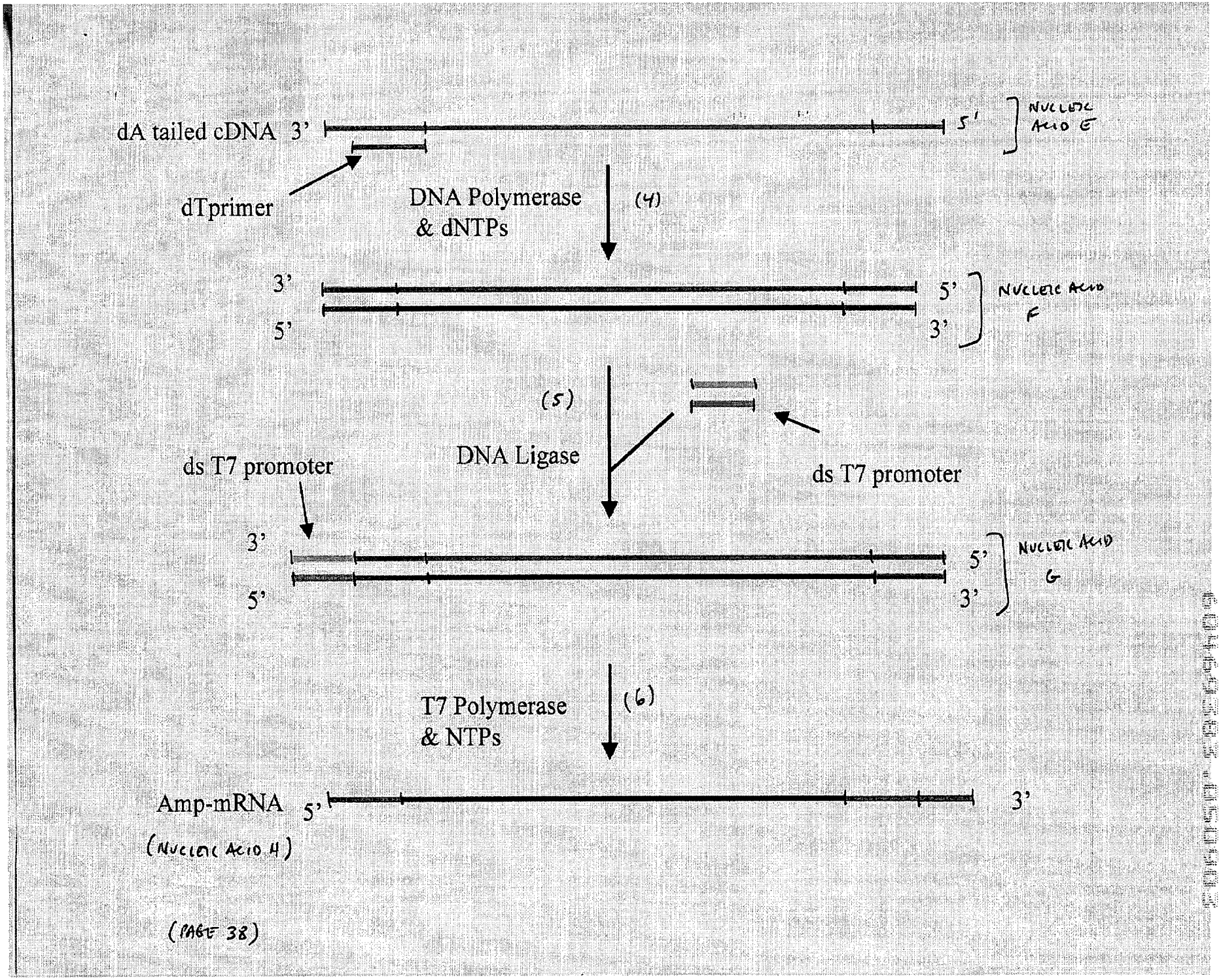
FIGURE 2 :

Blunt Ligation of T7 Promotor

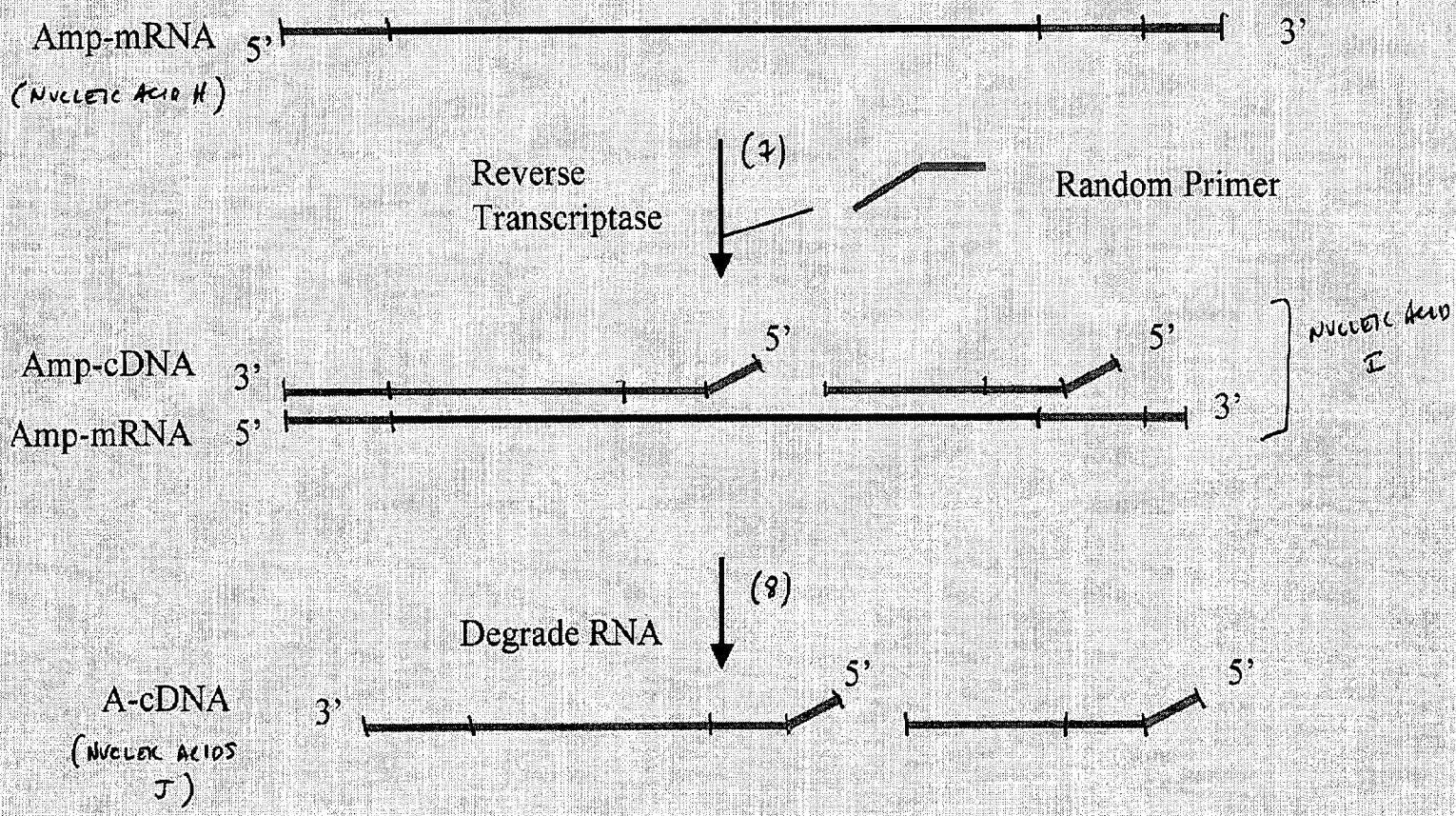
for use with Genisphere Array 350 or Array350RP Kits

Initial dT based priming follow by random prime target labeling

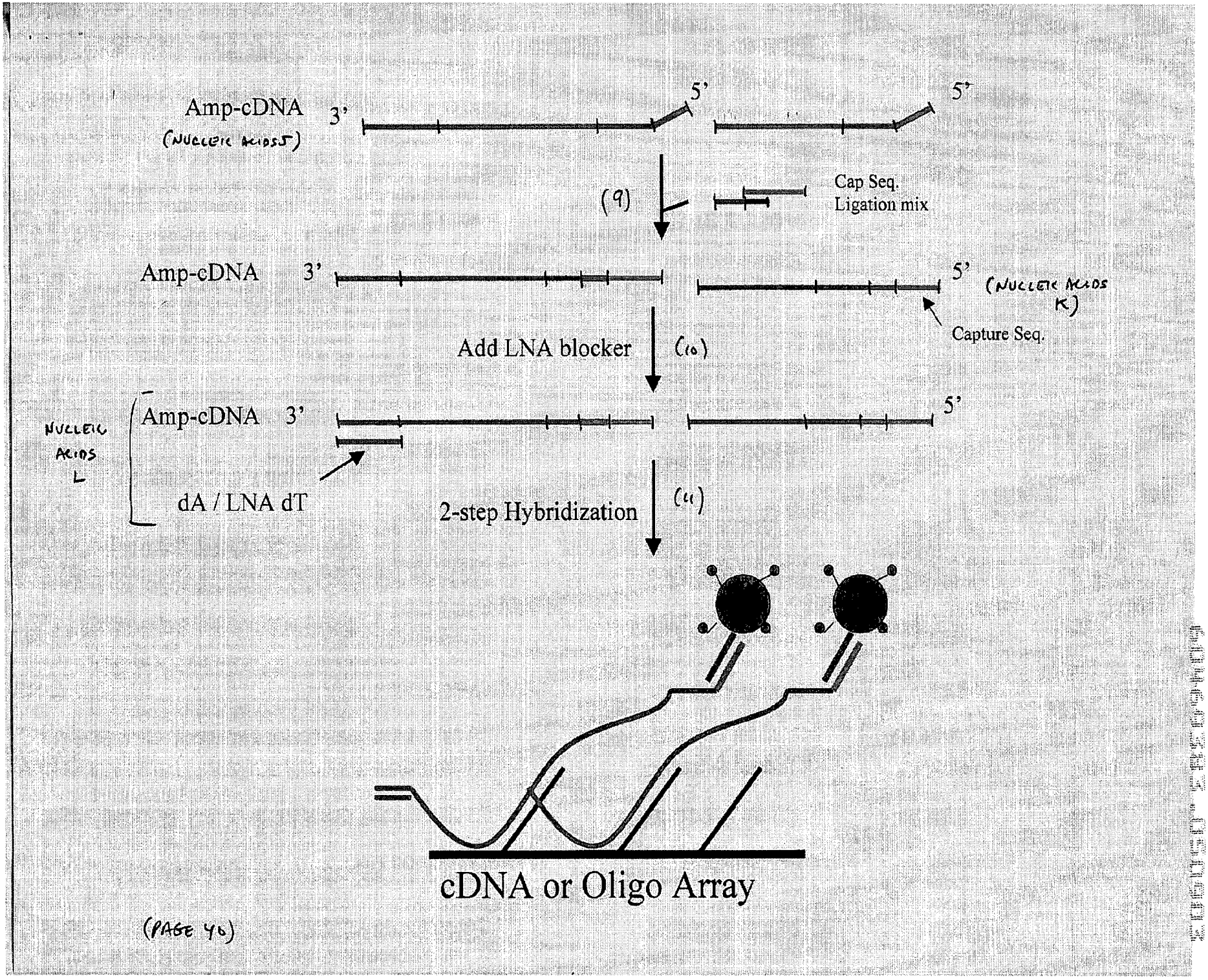



dA tailed cDNA 3'
5'
NUCLEIC ACID E
dTprimer
DNA Polymerase
& dNTPs
(4)
3'
5'
5'
3'
NUCLEIC ACID F
(5)
DNA Ligase
ds T7 promoter
ds T7 promoter
3'
5'
5'
3'
NUCLEIC ACID G
T7 Polymerase
& NTPs
(6)
Amp-mRNA 5'
3'
(NUCLEIC ACID H)

(PAGE 38)


Amp-mRNA
5'
3'
(NUCLEIC ACID H)
Reverse
Transcriptase
(7)
Random Primer
Amp-cDNA
3'
5'
5'
Amp-mRNA
5'
3'
NUCLEIC ACID
I
(8)
Degrade RNA
A-cDNA
(NUCLEIC ACIDS
J)
3'
5'
5'

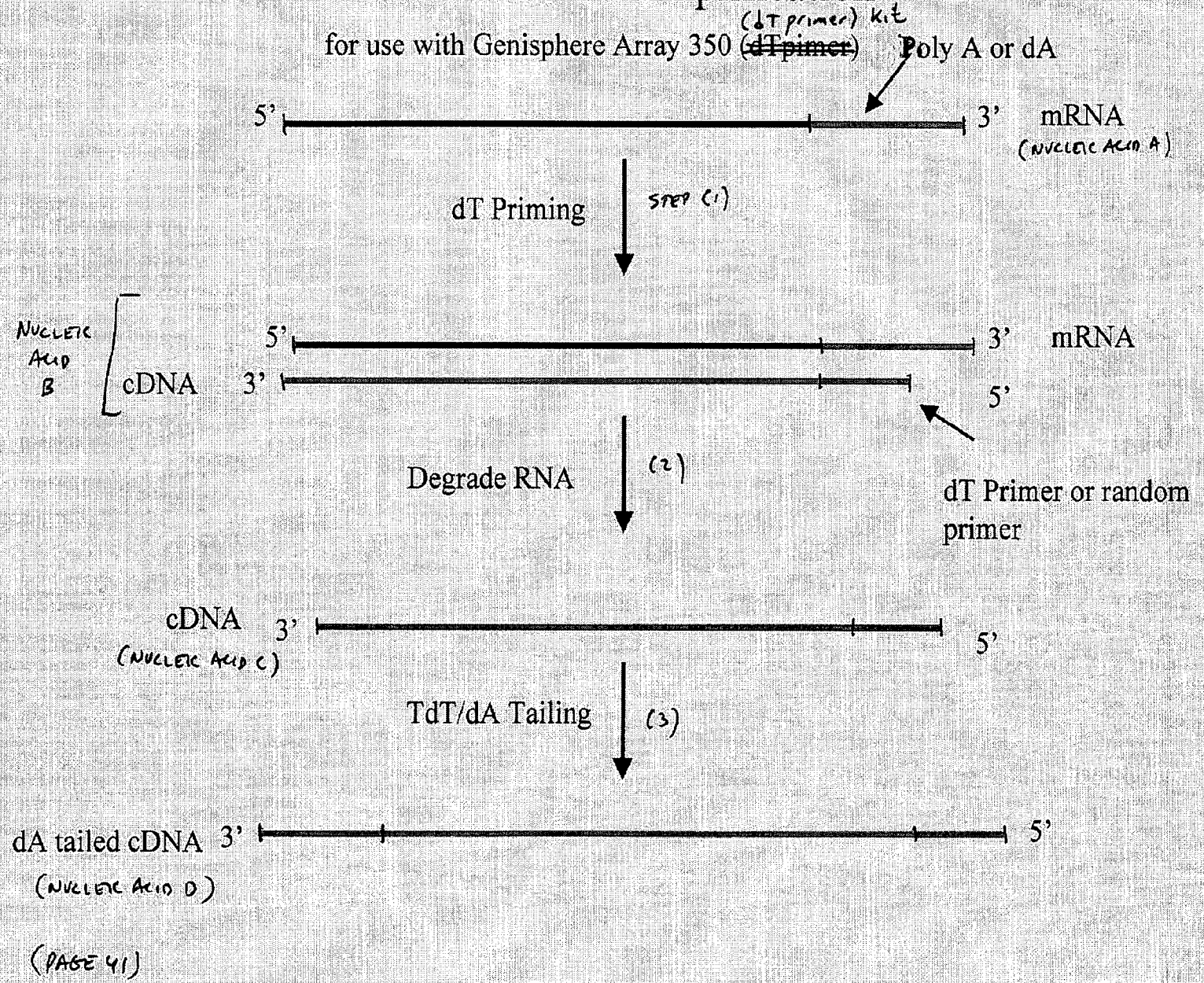
(PAGE 39)


Amp-cDNA
(NUCLEIC ACIDS J)
3'
5'
5'
(9)
Cap Seq.
Ligation mix
Amp-cDNA
3'
5'
(NUCLEIC ACIDS K)
Capture Seq.
Add LNA blocker
(10)
NUCLEIC ACIDS L
Amp-cDNA 3'
5'
dA / LNA dT
2-step Hybridization
(11)
cDNA or Oligo Array

(PAGE 46)

Figure 3: Complex Primer Alternative T7 Amplification

for use with Genisphere Array 350 (~~dTprimer~~) (dT primer) kit



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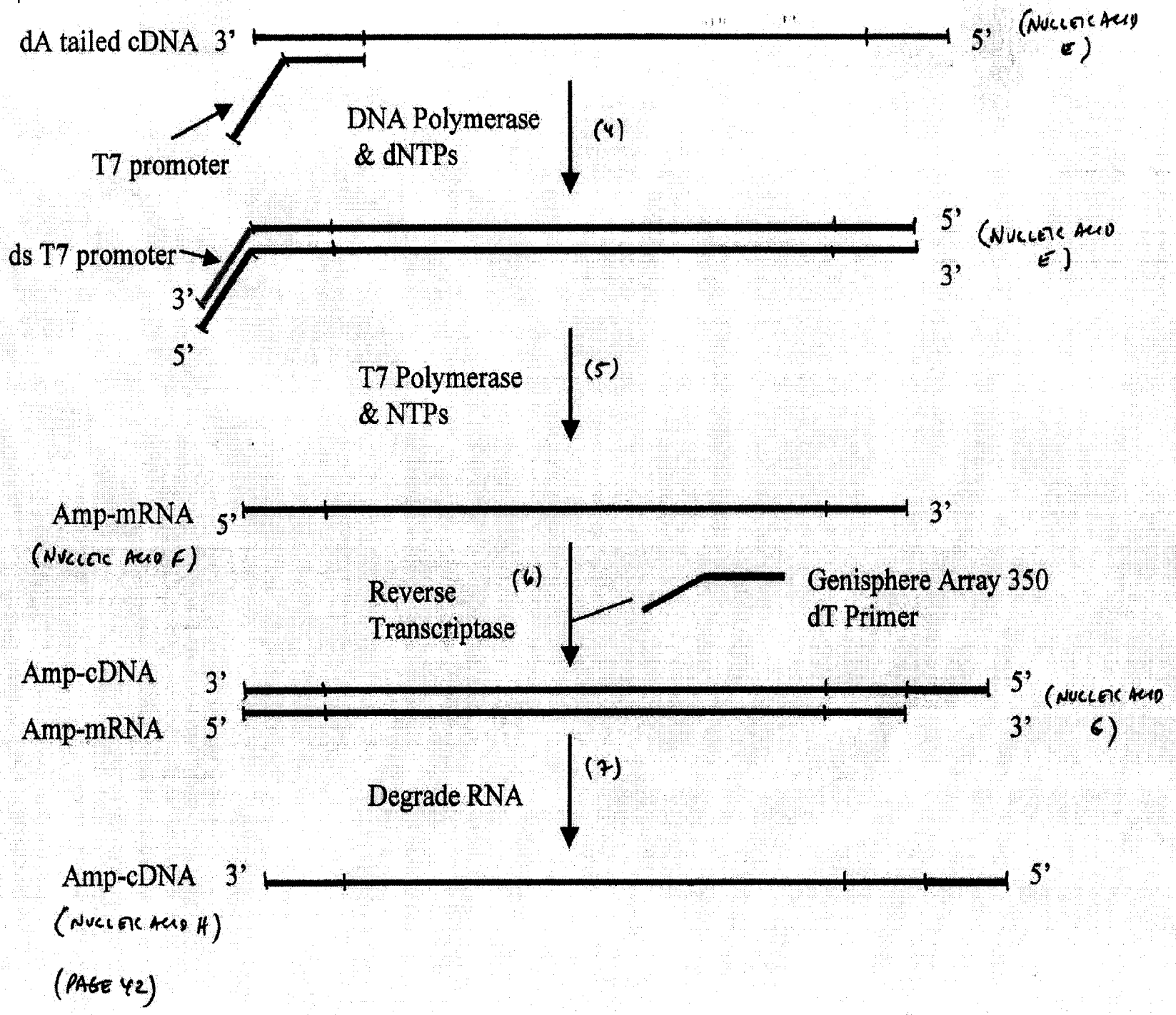
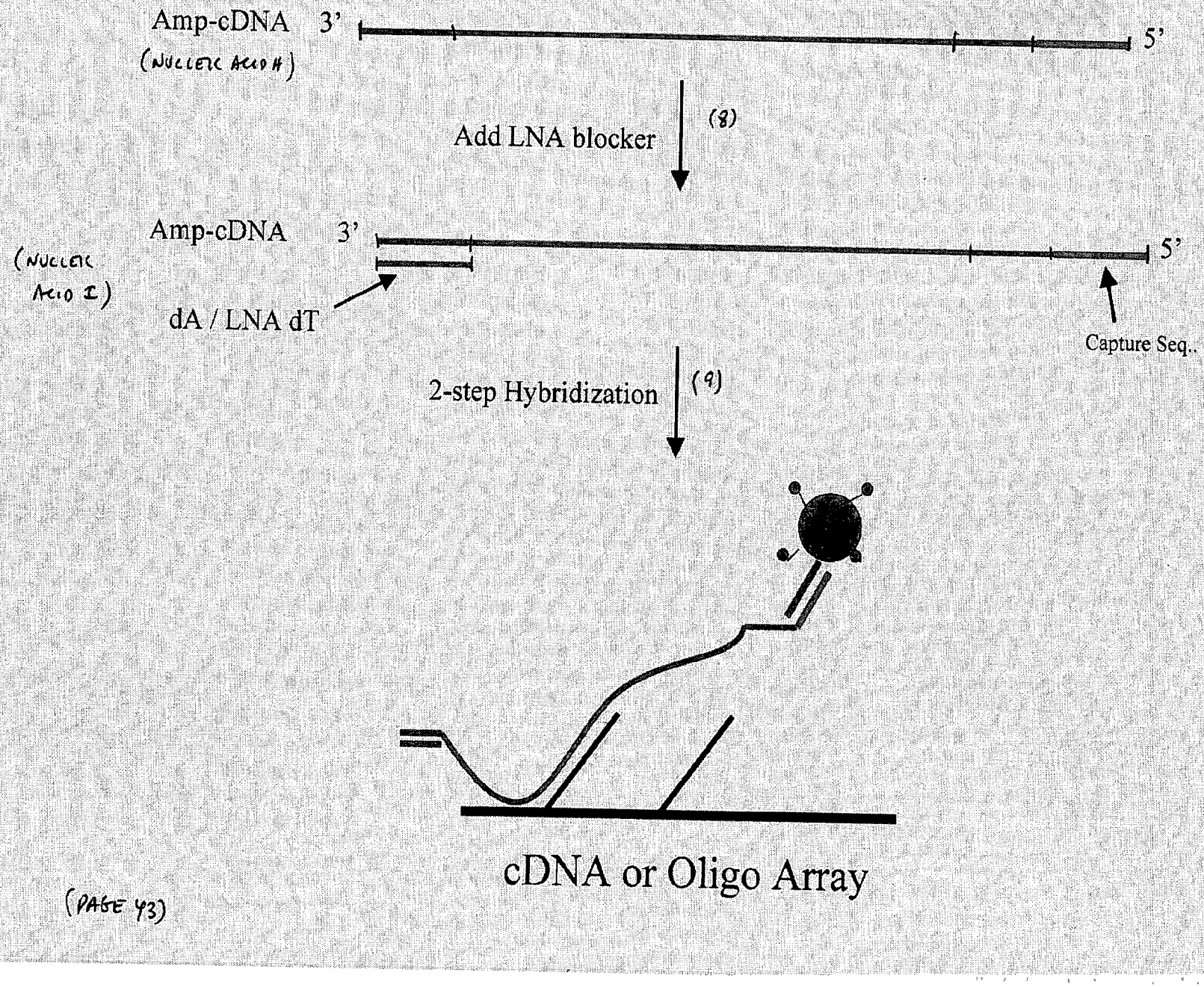

dA tailed cDNA 3'
5'
(Nucleic Acid E)
T7 promoter
DNA Polymerase
& dNTPs
(4)
ds T7 promoter
3'
5'
5'
3'
(Nucleic Acid E)
T7 Polymerase
& NTPs
(5)
Amp-mRNA 5'
3'
(Nucleic Acid F)
Reverse
Transcriptase
(6)
Genisphere Array 350
dT Primer
Amp-cDNA 3'
5'
Amp-mRNA 5'
3'
(Nucleic Acid G)
Degrade RNA
(7)
Amp-cDNA 3'
5'
(Nucleic Acid H)
(PAGE 42)


Amp-cDNA 3'
5'
(NUCLEIC ACID II)
Add LNA blocker
(8)
Amp-cDNA 3'
5'
(NUCLEIC ACID I)
dA / LNA dT
Capture Seq..
2-step Hybridization
(9)
cDNA or Oligo Array